ABSENCE OF RIBOSOMAL RNA SYNTHESIS IN THE ANUCLEOLATE MUTANT OF XENOPUS LAEVIS

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Few new ribosomes appear in the cytoplasm of embryos of *Rana pipiens* or *Xenopus laevis* (the South African "clawed toad") before the tail bud stage.[1] At this time the amount of cytoplasmic ribosomes begins to increase; this rise is correlated with an increase of protein in the high speed supernatant fraction as well as with the first appearance or increase of many enzymes. Soon after these events, the embryos develop a requirement for magnesium ions in the medium. Magnesium-starved embryos characteristically stop growing in length and die at early swimming stages (Shumway[2] stages 21–23 for *Rana pipiens*,[1] or Nieuwkoop-Faber[3] stage 40 for *Xenopus laevis*[4]). The magnesium requirement coincides with the onset of intense ribosome synthesis and presumably is based on the important role of magnesium ions in maintaining the integrity of the functional ribosome particle.

The study of ribosome synthesis during amphibian development has been extended utilizing the lethal anucleolate mutant of *Xenopus laevis* first described by Elsdale *et al.*[5] These workers discovered a heterozygote mutant with only one nucleolus (1-*nu*) in each cell, whereas wild-type *Xenopus laevis* have two nucleoli (2-*nu*) in the majority of their diploid cells. The progeny resulting from the mating of two heterozygotes (1-*nu*) fall into three groups having two, one, or zero nucleoli per cell. The ratio of these genotypes is 1:2:1, respectively,[5, 6] as expected of a typical Mendelian factor. The heterozygotes (1-*nu*) lack a secondary constriction ("nucleolar organizer") on one of two homologous chromosomes in diploid cells;[7] the two comparable chromosomes of the anucleolate homozygous mutants (0-*nu*) both lack this secondary constriction. The anucleolate mutant (0-*nu*) has numerous small nucleolar "blobs" instead of typical nucleoli, and both nuclear and cytoplasmic RNA have been shown histochemically to be lower in 0-*nu* embryos after hatching than in controls (1-*nu* and 2-*nu*).[8]

Development of 0-*nu* embryos is first retarded shortly after hatching.[5, 9] The mutant embryos become microcephalic and oedematous and die as swimming tadpoles before feeding. It was apparent that magnesium-starved embryos were to some extent phenocopies of the homozygous mutants (0-*nu*) since retardation of embryogenesis and growth occurred in both groups of embryos at about the same developmental stage (Fig. 1). The above data, as well as recent studies relating nucleolar function to ribosome synthesis, suggested that the anucleolate mutant might be incapable of synthesizing ribosomes and ribosomal RNA.

Material and Methods.—Radioactivity was introduced into developing embryos by incubation with $C^{14}O_2$ at pH 6.0.[10] The methods for measuring ribosome and DNA contents have been described previously.[1] Total RNA was isolated from frozen embryos after homogenization in 0.1 *M* sodium acetate pH 5.0 containing 4 μg/ml polyvinyl sulfate (a ribonuclease inhibitor prepared synthetically by the method of Bernfeld *et al.*)[11] and 0.5% sodium lauryl sulfate (Mann Research Co.). The homogenate was shaken for 5–10 min at 0°C with an equal vol of phenol. Nucleic acids were precipitated from the aqueous phase with 2 vol of ethanol and 0.1 vol of *M*

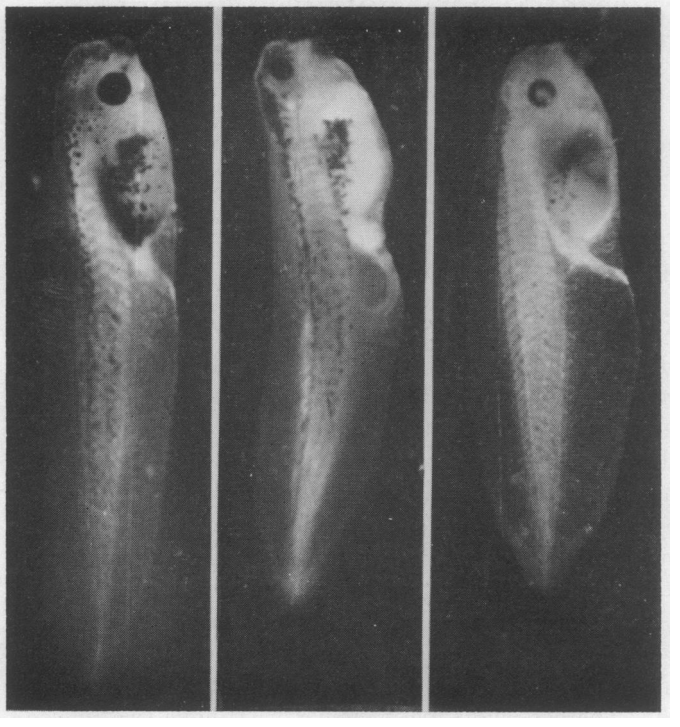

FIG. 1.—Comparison of control (left), anucleolate (middle), and magnesium - deficient (right) embryos of *Xenopus laevis*. These embryos are siblings that have developed for the same length of time. Initial symptoms characterizing the anucleolate mutant and magnesium deficiency syndrome are apparent.

sodium chloride, and the precipitate was dissolved in 0.01 *M* sodium acetate pH 5.0 containing 1 μg/ml polyvinyl sulfate. DNase I (5 μg/ml) and 10^{-3} *M* $MgCl_2$ were added and the solutions incubated for 10 min at 20°C. The RNA was further purified by two subsequent precipitations with NaCl-ethanol and the final precipitate drained of alcohol and dissolved in 1 ml of the 0.01 *M* sodium acetate-polyvinyl sulfate solution. Zonal sucrose gradient centrifugation[12] was performed in the SW-25 rotor of the Spinco Model L centrifuge for 14½ hr at 24,000 rpm. The nucleic acid solutions were layered over linear gradients of sucrose which varied from 20% to 5% and which contained 10^{-4} *M* versene and 0.01 *M* sodium acetate pH 5.0. Following centrifugation, the tubes were punctured and fractions collected for optical density measurement at 260 mμ and radioactivity determinations. Nucleic acids precipitated from each fraction by adding trichloroacetic acid to a concentration of 5% were caught on Millipore filters (HA) and dried. After phosphor was added, the filters were counted in a liquid scintillation counter.

Results and Discussion.—Absence of ribosomal RNA synthesis in the anucleolate mutant: Values for the RNA and DNA contents of anucleolate and control embryos are presented in Table 1. The most pronounced difference between the anucleolate *Xenopus* (0-*nu*) and the control mixture of 1-*nu* and 2-*nu* embryos is the reduced quantity of RNA and in particular the small amount of ribosomes in the 0-*nu* mutants.

TABLE 1
COMPARISON OF RNA AND DNA CONTENTS OF ANUCLEOLATE AND CONTROL *Xenopus laevis* EMBRYOS

	μg/embryo	
	Homozygous mutant	Control*
DNA†	0.88	0.97
Total RNA†	5.1	11.8
RNA contents of isolated ribosomes‡	3.2	5.4

* Analyses performed on a mixture of heterozygous (1-*nu*) and wild-type (2-*nu*) embryos.
† Control and mutant sibling embryos were at stages 40–41.[3]
‡ Control and mutant sibling embryos were at stages 38–40.[3]

The relatively small numbers of ribosomes present in the 0-*nu* embryos might have been synthesized entirely during oögenesis before meiotic reduction when the growing oöcytes were heterozygous for

nucleolus formation. Alternatively some ribosome synthesis might have occurred during embryogenesis. To distinguish between these possibilities, radioactive precursor was presented to the developing embryos during neurulation, when ribosomal RNA synthesis is known to have already begun in wild-type embryos;[4, 13] at this stage the 0-*nu* mutants are still developing normally and are morphologically similar to the control embryos. RNA was isolated 48 hr after termination of the radioactive incubation period so that ample time was allowed for complete utilization of the precursor and its incorporation into RNA. The density gradient centrifugation patterns of the total RNA isolated from 90 anucleolate (0-*nu*) mutants and 90 controls (a mixture of 1-*nu* and 2-*nu* embryos) are shown in Figure 2. The mutants contain about one

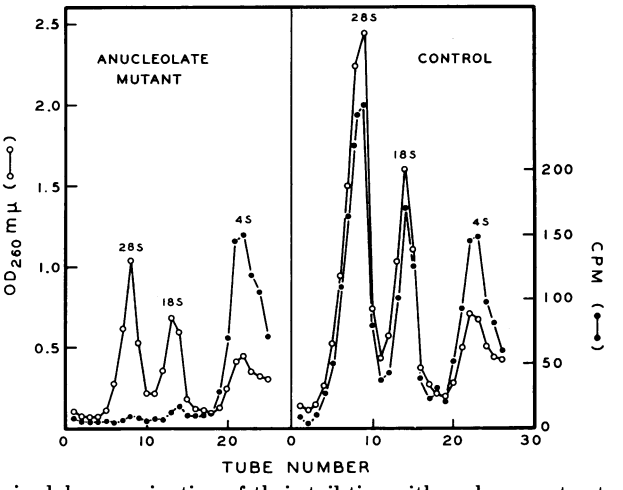

FIG. 2.—Sucrose density gradient centrifugation of total RNA isolated from 0-*nu* and control embryos. Two heterozygote (1-*nu*) adults were mated and the embryos allowed to develop to neurulation (Nieuwkoop-Faber[3] stages 14–18). At this time the embryos were incubated in a closed serum bottle at pH 6.0 with about 0.2 μc $C^{14}O_2$ for 20 hr at 18°C with mild shaking. By the end of this incubation period, development had proceeded to stages 26–28 (muscular response), and the mutant embryos were still indistinguishable grossly from the control embryos. The medium was changed, and the embryos continued development in nonradioactive tap water for 48 hr at 20°C (stages 40–41). The anucleolate mutants were recognized by examination of their tail tips with a phase contrast microscope and separated from the two control genotypes (1 *nu* and 2-*nu*). Both groups were then washed with distilled water and frozen at −20°C. The frozen embryos were packed in dry ice and flown from Oxford to Baltimore for chemical analysis. The bulk of the RNA (o—o) is represented by optical density measurements at 260 mμ. The RNA synthesized between neurula and muscular response stage is represented by the radioactive measurements (•—•).

half as much total RNA as the controls. This quantity (5 μg/embryo) is about the same as that found in the unfertilized egg of *Xenopus laevis*.[4] The control *Xenopus* embryos have synthesized radioactive 28S and 18S ribosomal RNA as well as 4S RNA. However, the 0-*nu* mutants have synthesized less than 5 per cent as much radioactive ribosomal RNA but about the same amount of 4S material as the control embryos. The radioactivity in the 4S region was eluted from the Millipore filters with dilute NH_4OH, made 0.3 *N* with KOH and incubated overnight at 37°C. About 80 per cent of the radioactivity in both mutant and control samples was presumably RNA since it was rendered acid-soluble by this treatment. The remaining alkali-resistant radioactivity was solubilized by hot TCA and probably was partially degraded radioactive DNA.

In these 0-*nu* mutants the ribosomal RNA made during oögenesis has persisted, and the embryos are incapable of synthesizing new ribosomal RNA. Since these embryos develop normally to early swimming stages, it can be concluded that *Xenopus* embryos do not *need* new ribosomal NRA until after this stage of development.

Synthesis of rapidly labeled RNA by the anucleolate mutant: To define the classes of rapidly labeled RNA synthesized by the mutant, RNA was isolated immediately

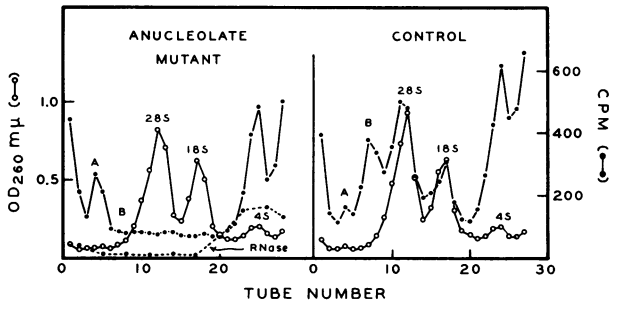

FIG. 3.—Rapidly labeled RNA of mutant (0-*nu*) and control *Xenopus* embryos. Previously separated 0-*nu* mutants and control (1-*nu* and 2-*nu*) embryos (32 embryos each) at stage 27–28 were placed in a 2 ml vial with 1 ml of preboiled Holtfreter-M^{++} medium[1] containing 0.3 *M* sodium phosphate pH 6. After gently blowing CO_2-free air over the medium for 5 min, the vials were sealed with rubber injection caps, and 25 μc of $Na_2C^{14}O_3$ dissolved in 0.01 *M* NaOH was injected into each bottle. The vials were gently shaken for 2 hr at 21°C, then cooled, and the embryos washed with cold distilled H_2O. Nonradioactive RNA (0.8 mg) isolated from *Xenopus* oöcytes was added to each group of embryos and the total RNA purified. The purified RNA was dissolved in 1.5 ml, and 1.0 ml (equivalent to RNA from 20 embryos) was centrifuged. The remaining 0.5 ml of the mutant RNA preparation was made 0.1 *M* with tris buffer pH 7.2 and incubated in a total volume of 1 ml with 20 μg of pancreatic RNase for 10 min at 20°C. Values for the RNase-treated preparation have been corrected for volume and tube number so that they are directly comparable with the untreated RNA.

after a 2-hr incubation with labeled precursor (Fig. 3). Because of the small number of embryos used in this experiment, purified carrier RNA (unlabeled) was added at the beginning of the isolation procedure, so that the optical density peaks of the carrier RNA serve as reference markers for the three classes of bulk RNA, i.e., 28S, 18S, and 4S RNA.

Experiments by Scherrer *et al.*[14] and Perry[15] indicate that the 28S and 18S ribosomal RNA molecules are both derived from larger precursor molecules. Radioactive label appears first in these rapidly sedimenting precursors and only later in 28S and 18S RNA. The results plotted in Figure 3 demonstrate that 2 hr after addition of radioactivity the control already has synthesized 28S and 18S RNA as well as at least two distinct peaks of heavier RNA (labeled *A* and *B*). In contrast, the mutant embryos not only failed to synthesize typical 28S and 18S ribosomal RNA but also lack the heavy precursor RNA that sediments in region *B* of Figure 3 (about 35S). Yet heavier classes of RNA (type A and even more rapidly sedimenting molecules) have been synthesized by the mutant, as well as heterogeneous RNA that sediments throughout the gradient solution. This latter observation is more evident when the sedimentation pattern of rapidly labeled mutant RNA is compared before and after ribonuclease digestion (Fig. 3).

The rapidly labeled RNA synthesized by the mutant is most probably "messenger" RNA. This conclusion is based on the fact that in the mutant all the radioactive RNA sedimenting more rapidly than 4S can be recovered associated with the purified ribosomes of the mutant. When isolated by this technique, the rapidly labeled RNA is degraded to molecules having sedimentation constants between 4 and 18S. The base composition of this heterogeneous RNA labeled with P^{32}, which has been isolated in association with purified ribosomes of normal *Xenopus* embryos, is invariably DNA-like.[4]

The nucleolus as the site of ribosomal RNA synthesis: In control *Xenopus* embryos, although other classes of RNA are synthesized at earlier stages, new *ribosomal* RNA synthesis is not detectable until gastrulation,[4] the same stage that definitive nucleoli first become visible cytologically. (The many small "blobs" seen in

blastula nuclei do not seem to be equivalent to "definitive" nucleoli.) Although synthesis of ribosomal RNA begins at gastrulation, the quantity of this newly synthesized RNA remains small when compared to the RNA already present in the unfertilized egg. It is only after hatching that the total RNA content of wild-type *Xenopus* embryos begins to increase significantly.[4] The absence of typical nucleoli at very early stages of development has been reported for other amphibia[16] as well as other developing organisms.[17] Furthermore, Beermann[18] has described developmental arrest in anucleolate recombinants resulting from the mating of two different species of the dipteran, *Chironomus*. The relationship between nucleolar function and ribosome synthesis has been suggested by several observations including electron microscopy,[19, 20] base composition analyses,[20] and radioautographic studies.[15] The close correlation of the time of ribosomal RNA synthesis with the appearance of definitive nucleoli in *Xenopus* and particularly the simultaneous absence of both ribosomal RNA synthesis and normal nucleoli in the 0-*nu* mutant support this relationship.

Difference in nucleotide composition between 28S and 18S RNA: This single mutation prevents the formation of both 28S and 18S RNA molecules (Fig. 2); however, evidence has been presented suggesting that the structure of these two molecules is determined by different gene loci. Yankofsky and Spiegelman[21] have shown that 23S and 16S ribosomal RNA of bacteria hybridize independently with homologous bacterial DNA. Thus, the two molecules must have different nucleotide sequences each complementary to a distinct region of the bacterial DNA. In *Xenopus*, the 28S and 18S ribosomal RNA have different base compositions. Table 2 contains analyses for 28S, 18S, and 4S RNA's separated by density gradient centrifugation

TABLE 2

NUCLEOTIDE COMPOSITION OF 28S AND 18S RIBOSOMAL RNA AND 4S RNA PURIFIED FROM *Xenopus laevis* EGGS AND EMBRYOS

Approximate S Value	Unfertilized Eggs 28	18	4	Stage 45 Embryos 28	18	4
AMP	17	22	20	18	22	21
GMP	37	31	28	37	31	31
CMP	30	29	32	28	29	30
UMP	16	18	20	17	18	18
% GC	67	60	60	65	60	61

Following density gradient centrifugation, the RNA was precipitated from the sucrose solutions with cold TCA, washed with ethanol, and hydrolyzed in NHCl at 100°C for 1 hr. Base composition was determined following chromatography in the isopropanol:HCl solvent described by Wyatt.[22]

from RNA purified from ovarian eggs and embryos of *Xenopus laevis*. The 28S RNA has a significantly higher G-C content than the 18S RNA. There is also a difference in base composition between the 23S and 16S ribosomal RNA's of different bacteria[23] as well as the 28S and 18S ribosomal RNA's of chick embryos.[24]

Quantitative regulation of ribosomal RNA gene activity: The rates of ribosomal RNA synthesis in 1-*nu* and 2-*nu* embryos were compared. The results shown in Table 3 demonstrate that all three classes of RNA molecules are synthesized at comparable rates by the heterozygote and homozygous wild-type embryos. Furthermore, the synthesis of 28S and 18S RNA is coordinate since their specific activities are the same. Thus, the haploid complement of ribosomal RNA genes in the heterozygote must produce twice as much ribosomal RNA as do the same genes in the wild-type homozygote. It is of interest to note that the combined

TABLE 3

RNA SYNTHESIS BY 1-*nu* AND 2-*nu* *Xenopus laevis* EMBRYOS

	1-*nu*	2-*nu*
Total RNA μg/embryo	9.1	10.8
	CPM/μg RNA	
28S	0.45	0.38
18S	0.39	0.37
4S	0.95	0.89

The same protocol that is described in the legend to Fig. 2 was followed. Thus, sibling embryos were made radioactive at the same stage and under the same conditions. The radioactive 1-*nu* and 2-*nu* embryos were separated, and the specific activity of 28S, 18S, and 4S RNA was calculated following density gradient centrifugation of the purified RNA.

volume of the 2 nucleoli in 2-*nu* embryos is the same as that of the single nucleolus in 1-*nu* heterozygotes.[6, 25]

Genetic basis of the anucleolate condition: The mutation affecting nucleolar number behaves as a single Mendelian factor and results in a cytologically visible alteration on one chromosome, i.e., in "the nucleolar organizer" region.[7] The defect when homozygous does not alter nucleic acid metabolism generally, but specifically prevents the synthesis of both molecular species of ribosomal RNA. Thus DNA (Table 1), 4S RNA (Fig. 2), and rapidly labeled high molecular weight heterogeneous RNA (Fig. 3) are all synthesized by the mutant 0-*nu* embryos. Furthermore, the relative synthesis of both 28S and 18S RNA is the same (Table 3), even at different developmental stages when ribosomal RNA is formed at widely different rates.[4]

Spiegelman[26] and Scherrer *et al.*[14] have reasoned that closely linked genes (perhaps whole operons[27]) might be transcribed as single large RNA molecules ("polycistronic" RNA[28]) which are subsequently degraded specifically to smaller and, in the case of ribosomal RNA, stable subunits. If adjacent 28S and 18S genes were transcribed together as a single molecule, such a large precursor would be expected to have a molecular weight of about 2–3 $\times$ 10^6 with a sedimentation constant of approximately 35S. This is about the sedimentation constant of the ribosomal RNA precursor (Region *B*, Fig. 3). This hypothesis accounts for the fact that large precursor molecules give rise to smaller ones as well as providing a molecular basis for coordinate expression of the several genes of an operon. Since the 28S and 18S ribosomal RNA molecules function together as components of a single structure, the ribosome, it is reasonable that their synthesis should be controlled together.

Two general mechanisms adequately account for the characteristics of the anucleolate mutant. The anucleolate condition might be considered as a primary defect of nucleolus formation which secondarily results in the absence of ribosomal RNA synthesis. The alternative hypothesis would have the primary defect of preventing ribosomal RNA synthesis. This latter idea suggests that the nucleolus marks the location of ribosomal RNA and ribosome synthesis in the nucleus, and the presence of the nucleolus is secondary to these synthetic processes. The comments to follow do not distinguish between these two general possibilities but serve to analyze pertinent genetic mechanisms in the light of the data presented in this report.

The consequences of the anucleolate mutation cannot be explained by the alteration of a "repressor or activator" substance[27] that might circulate in the nucleoplasm and inhibit or activate the structural genes for ribosomal RNA. If a substance exists in the nucleoplasm which regulates expression of the ribosomal RNA genes, it would be expected to act equally on *both* nucleolar organizers of a diploid cell (unless it only acts in the immediate vicinity of its own synthesis). Thus an altered repressor such as a "superrepressor"[29] would be *dominant* resulting

in an *anucleolate heterozygote* since the altered repressor would inhibit expression of both nucleolar organizers in diploid cells. If, on the other hand, expression of nucleolar organizers required the constant presence of an "activator" substance of endogenous origin, the nonproduction of such a substance would have a *recessive* effect, i.e., the heterozygote embryos (1-*nu*) would contain two nucleoli in each cell just as the control embryos. *In fact, the anucleolate mutation has resulted in nonfunctional gene loci which remain nonfunctional even in the presence of their normal alleles.*

It is highly probable that many genes are involved in the synthesis of ribosomal RNA. Both in bacteria[21] and in mice[30] the ribosomal RNA is complementary to 0.3 per cent of the DNA (0.6% of the nucleotide pairs). It seems likely that a similar proportion will be present in the *Xenopus laevis* genome since ribosomal RNA can constitute such a large fraction of the gene product. If so, there must be thousands of separate gene loci for each class of ribosomal RNA.

If the DNA for ribosomal RNA is distributed amongm any chromosomes, it is difficult to imagine how its products can be concentrated into one nucleolus, while still accounting for the biochemical and cytological properties of the 0-*nu* and 1-*nu* embryos. On the other hand, the entire complement of DNA for ribosomal RNA may be adjacent on a single chromosome. Since there are 18 haploid chromosomes in *Xenopus* the ribosomal region would occupy roughly 10 per cent of one of them, presumably the one containing the nucleolar organizer.

A single deletion would account for the results but would require the loss of an extremely large piece of DNA. Alternatively, an operator mutation[27] would account for the features of the anucleolate mutation. However, this would imply that a single operator locus could control the expression of thousands of genes.

Any mechanism invoked to explain ribosomal RNA synthesis must account for the fact that the activity of the entire complement of genes determining ribosomal RNA structure can be restricted by a single mutation.

Summary.—A mutation in *Xenopus laevis* that prevents the formation of a normal nucleolus at the same time prevents the synthesis of 28S and 18S ribosomal RNA as well as high molecular weight ribosomal RNA precursor molecules. DNA, 4S RNA, and rapidly labeled heterogeneous RNA are synthesized by the anucleolate mutant. Anucleolate mutants survive until the swimming tadpole stage and show normal differentiation of all the main cell types despite their inability to synthesize new ribosomal RNA. Homozygous mutants (0-*nu*) and control embryos conserve the ribosomes made during oögenesis and associate rapidly synthesized RNA with these old ribosomes.

The 28S and 18S ribosomal RNA's differ in base composition and are probably products of different genes; yet their synthesis is coordinate. In the heterozygous (1-*nu*) embryos, the wild-type genes regulate to produce twice as much 28S and 18S ribosomal RNA as do the same genes when present in homozygous wild-type individuals. Since the activity of the entire complement of genes determining ribosomal RNA structure can be curtailed by a single mutation, it is suggested that these genes are under common control and located at the "nucleolar organizer" site of a single chromosome.

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